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Red cell 2,3-DPG levels have been augmented for improved hemoglobin-oxygen dissociation during blood storage under blood banking conditions by a variety of metabolic manipulations. An optimal pH for both 2,3-DPG and ATP maintenance was determined. Addition of pyruvate, dihydroxyacetone, ribase, xylase, and xylitol were especially useful.

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DEPARTMENT OF THE ARMY
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May 5, 1988

REPLY TO
ATTENTION OF
SGRD-RMI-S

SUBJECT: Review of Draft Final Report, April 1, 1988, for Contract No. DADA17-72-C-2005

Ben R. Dawson, M.D.
University of Maryland
737 W. Lombard Street
Baltimore, Maryland 21201

Dear Dr. Dawson:

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HEMOGLOBIN FUNCTION AND RED CELL METABOLISM IN STORED BLOOD

Final Report

Ben R. Dawson, M.D.

April 1, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DADA17-72-C-2005

University of Maryland
737 W. Lombard Street
Baltimore, Maryland 21201

FOREWORD

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

The goal of blood preservation research is to improve the viability and the function of stored red blood cells. Blood in CPD (citrate-phosphate-dextrose) preservative is currently licensed for up to 3 weeks of storage, whereas CPD-adenine (CPD-A) is licensed for up to 5 weeks. Each additional week of blood storage capability reduces blood outdating and wastage by an estimated 50%.

In addition to extending blood storage time, the quality of blood that is transfused is of essential importance. ATP (adenosine triphosphate) is currently the primary criterion for blood quality and enough ATP for a 70% post-transfusion cell survival rate is considered necessary. 2,3-biphospho-glycerate (2,3-DPG), however, is depleted in blood well before ATP reaches critical levels with CPD-A preservative. 2,3-DPG is essential as an effector in hemoglobin oxygen dissociation. In patients with impaired compensatory mechanisms for low 2,3-DPG levels, such as cardiac surgery patients, patients in septic shock, or patients with severe trauma who require massive transfusions, adequate levels of 2,3-DPG are of utmost importance.

Several tactics have been taken to improve maintenance of 2,3-DPG in blood. Although many have been successful, few have been able to maintain 2,3-DPG without a concomitant decrease in ATP maintenance. ATP is necessary for cell viability and membrane integrity. Reasons for the decrease in ATP are evident in the glycolytic metabolism of erythrocytes. In order to produce 2,3-DPG through the Rapoport-Luebering shunt, an ATP-producing step must by bypassed, causing no net synthesis of ATP in the cell. Most attempts by our lab to resolve this problem have included adding additional intermediary

metabolites to whole blood in CPD-A preservative. However, several studies were conducted into the ideal glucose and pH conditions in CPD and CPD-A preservatives.

The glucose requirements of soft- and hard-packed cells were studied in order to ascertain the ideal glucose concentration. Glucose is adequate for 5 weeks of storage. Nevertheless, more was concluded to be beneficial for additional storage in soft-packed cells, although not in whole blood. Hard-packed cells benefit from 175% to 200% of the normal concentration of glucose in CPD-A, particularly after 42 days.

A higher pH is detrimental to ATP maintenance and beneficial to 2,3-DPG maintenance. A pH between 5.5 and 6.0 is recommended in CPD for higher 2,3-DPG levels without significant losses in ATP. 5.6 is the ideal pH in that range. The adverse effect on 2,3-DPG at low pH's is reversed by the addition of ionsine and methylene blue (MB).

Inosine and MB maintain higher to near normal levels of 2,3-DPG with increasing inosine concentrations, although this increase also lowers ATP levels. 10 mM inosine is necessary for maintenance of normal 2,3-DPG levels. MB has the effect of stimulating the pentose phosphate pathway by the oxidation of NADPH to NADP; nonetheless, this effect appears to be dependent on the presence of inosine. Inosine is a nutrient that is eventually catabolized to Ribose-5-P, a metabolite in the pentose phosphate pathway. Yet, the effect of MB on CPD-A preservatives is minimal in aiding 2,3-DPG or ATP maintenance. Phosphate, in 10 or 20 mM concentrations, aids inosine remarkably in 2,3-DPG and ATP maintenance. Normal levels of both have been observed after 6 weeks with an inosine-phosphate preservative. Inosine, which supplies a ribose, and ribose, a natural nutrient that enters the pentose

phosphate pathway, are quite successful in benefiting 2,3-DPG, but are detrimental to ATP levels. Ascorbate has largely the same effect as ribose when combined with inosine. Together, the effects are greater than when separate. MB does not affect a preservative with inosine and ascorbate.

A disadvantage with inosine is the production of hypoxanthine in the splitting of this nucleoside that yields ribose-1-P, a precursor to ribose-5-P. Hypoxanthine, in vivo, causes an accumulation of uric acid that can be toxic. Inosine, therefore, is not useful in ordinary blood storage, but instead has potential in storage where the supernatant is removed after incubation as in the optional additive solutions like Adsol$^{(R)}$ and SAG.

Methylene blue, used in concentrations of 10^{-6} M in these experiments, has not shown toxicity with the larger amounts used clinically for the treatment of methemoglobinemia. The effect of MB with ribose appears to be an oxidation of NADH to provide the NAD needed for the G-3-P step in glycolysis. The large amount of G-3-P produced from ribose can then increase 2,3-DPG. ATP, however, is decreased rapidly, possibly as a result of the initial ATP-utilized in phosphorylation of ribose without a subsequent replenishment of ATP. Although ribose exhibits an optimum concentration of 2.5 mM, MB removes this optimum. Ribose alone has little effect on either 2,3-DPG or ATP levels. Nevertheless, ribose with phosphate increases maintenance of ATP to normal levels and maintains 2,3-DPG significantly better than the CPD-A control, although not at normal levels. As with MB and inosine, ribose has been used intravenously. Hypoglycemia is induced by ribose, although doses of 5 grams produce only a mild degree, whereas even a 10 mM concentration in a blood preservative would provide only 0.75 grams of ribose per unit of whole blood.

Other five-carbon sugars besides ribose have been studied by our lab as potential blood preservative additives. Among these were xylose and with its alcohol, xylitol. Xylose enters the pentose phosphate pathway through xylulose and its ATP-utilizing phosphorylation to xylulose-5-P. Xylose has only a small effect in benefiting 2,3-DPG and lowering ATP maintenance. However, xylitol first reduces NAD to NADH. Because of this, it has an initial moderate lowering in ATP; however, its ATP values do not vary significantly from the CPD-A control throughout storage as they actually increase in later weeks. Xylitol significantly benefited 2,3-DPG maintenance. Its use in a blood preservative is limited by a possible connection with oxalate production. Xylitol has been used as parenteral nutrition in patients, but calcium oxalate crystals were observed. Levels needed in a blood preservative would be lower than that implicated in oxalate production. Therefore, xylitol has a potential as a blood storage preservative because of its ability to better maintain 2,3-DPG without significant detriment to ATP levels.

Several six-carbon monosaccahrides were compared to dextrose in their ability to improve blood storage. Galactose was not successful; however, fructose and mannose both somewhat improve 2,3-DPG maintenance compared to the dextrose preservative. Mannose is slightly worse, whereas fructose is slightly better for ATP maintenance. Three disaccharides--lactose, maltose, and sucrose--are poor in 2,3-DPG and ATP maintenance.

An extensive amount of research has been conducted on pyruvate, particularly in conjunction with other additives. Alone, pyruvate has a dramatic effect on maintaining 2,3-DPG, but also causes a large concurrent drop in ATP levels. With dihydroxyacetone (DHA), pyruvate has an even greater

effect on 2,3-DPG, but ATP levels are not improved. Nor is phosphate able to reverse pyruvate's detrimental effect on ATP like it is able to do with inosine, DHA, and ribose. Phosphate, however, does not harm 2,3-DPG maintenance in pyruvate or pyruvate and DHA preservatives. Pyruvate and ascorbate are quite beneficial for 2,3-DPG, as they are together with DHA and phosphate. However, ATP levels in these pyruvate-containing preservatives are too low to be sufficient in sustaining erythrocyte viability.

DHA has a lesser, but similar effect on 2,3-DPG and ATP compared to pyruvate. DHA is able to increase 2,3-DPG levels in a rejuvenation study, although not as much as pyruvate unless combined with MB. Little ATP is regenerated with these preservatives. 10 mM phosphate, however, does vastly improve ATP maintenance in a preservative containing 60 mM DHA. ATP is maintained at levels comparable to the CPD-A control. 2,3-DPG is maintained quite well with a DHA-phosphate preservative. DHA, pyruvate and ascorbate have not exhibited any toxic effects. DHA and ascorbate are beneficial for 2,3-DPG but detrimental to ATP maintenance. DHA and ascorbate, when combined with phosphate, improve both 2,3-DPG and ATP levels compared to a preservative without phosphate, but still worsen ATP maintenance relative to the CPD-A control.

Ascorbate (or vitamin C), at a 10 mM concentration, maintains near normal 2,3-DPG levels for 6 weeks of storage with less ATP maintenance than the CPD-A control. Both the natural L and the unnatural D isomers of ascorbate maintain 2,3-DPG equally well. Ascorbate's mode of action has been theorized from studies conducted in our lab. Carbon monoxide does not alter ascorbate's effect, suggesting that ascorbate's major influence is not through a reaction with the oxygen in oxyhemoglobin. Dehydroascorbate has much more of an effect

than ascorbate in 2,3-DPG maintenance. This suggests that ascorbate acts through oxidative rather than reductive processes since ascorbate and dehydroascorbate are reducing and oxidative agents, respectively. Ascorbate must be reduced in the erythrocyte to dehydroascorabte in order for ascorbate to exhibit its similar, though lesser, effect compared to dehydroascorbate.

Ascorbate was studied with N-ethylmalemide (NEM) and iodoacetate (IA) which are sulfhydryl inhibitors. They enhance ascorbate's effects on erythrocytes. IA prevents much of the red blood cell's metabolism. Glutathione (GSH) has a minimal effect on ascorbate-containing preservatives. From various combinations of ascorbate, NEM, IA, and GSH, it was theorized that ascorbate aids 2,3-DPG maintenance in the red blood cell by oxidizing NADH, and is not dependent on sulfhydryl or glutathione systems. Adverse effects on ATP maintenance appear to be dependent on the erythrocyte's sulfhydryl system. In addition, glucose is spared with L-ascorbate, suggesting that L-ascorbate is used as a nutrient.

In improving the currently-used CPD-A preservative, it is necessary to add a compound or metabolite that improves 2,3-DPG without harming ATP maintenance, as well as one that is non-toxic in levels that would be used. A preservative exhibiting such capabilities is DHA with phosphate. 60 mM DHA with 10 mM phosphate in a CPD-A preservative clearly demonstrates a vast improvement in 2,3-DPG maintenance while it maintains ATP at levels equal to or better than a CPD-A control. After 6 weeks, 2,3-DPG levels for DHA and phosphate are approximately 5 times the control. Furthermore, no toxicity has been reported with DHA, and phosphate is already used in CPD-A, although in a smaller concentration. Other preservatives studied still warrant further study on their potential in benefiting cell viability and function at safe

levels for transfusion. Pyruvate's adverse effect on ATP is not able to be reversed by the addition of other additives. Methylene blue has little effect on its own and although it can benefit 2,3-DPG in combinations with other metabolites, it does not improve ATP levels. Fructose and mannose are beneficial but limited in their results. Xylitol causes higher levels of 2,3-DPG without harming ATP and may be practical if the 40-60 mM concentrations are not conducive to oxalate production. Ribose, and particularly inosine are quite advantageous when used with phosphate in their increase of both 2,3-DPG and ATP maintenance compared to CPD-A alone. Ribose ought to be safe in the necessary 2.5 mM concentration with 10 mM phosphate although inosine would require the removal of the supernatant. Ascorbate has not been demonstrated to be sufficient in ATP maintenance, although it has not yet been examined with phosphate. CPD-A with DHA and phosphate is a promising and successful alternative to the presently-used CPD-A preservative. Xylitol, possibly ascorbate with another metabolite, and inosine and ribose each with phosphate also demonstrate potential as additives to CPD-A preservative that would greatly benefit many patients that require normal erythrocyte levels of 2,3-DPG.

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